

An Energy-Minimized Casein Submicelle Working Model

Received January 12, 1994

To develop a molecular basis for structure-function relationships of the complex milk protein system, an energy-minimized, three-dimensional model of a casein submicelle was constructed consisting of κ -casein, four α_{s1} -casein, and four β -casein molecules. The models for the individual caseins were from previously reported energy-minimized, three-dimensional structures. Docking of one κ -casein and four α_{s1} -casein molecules produced a framework structure through the interaction of two hydrophobic antiparallel sheets of κ -casein with two small hydrophobic antiparallel sheets (residue 163–174) of two preformed α_{s1} -casein dimers. The resulting structure is approximately spherically symmetric, with a loose packing density; its external portion is composed of the hydrophilic domains of the four α_{s1} -caseins, while the central portion contains two hydrophobic cavities on either side of the κ -casein central structure. Symmetric and asymmetric preformed dimers of β -casein formed from the interactions of C-terminal β -spiral regions as a hinge point could easily be docked into each of the two central cavities of the α - κ framework. This yielded two plausible energy-minimized, three-dimensional structures for submicellar casein, one with two symmetric β -casein dimers and one with two asymmetric dimers. These refined submicellar structures are in good agreement with biochemical, chemical, and solution structural information available for submicellar casein.

KEY WORDS: Casein structure; protein functionality; milk proteins.

1. INTRODUCTION

The caseins occur in bovine milk as colloidal complexes of protein and salts, commonly called casein micelles. Removal of calcium is thought to result in the dissociation of this micellar structure into noncolloidal protein complexes called submicelles (Farrell, 1988). These submicelles consist of four proteins, α_{s1} -, α_{s2} -, β -, and κ -casein, in the ratios of 4:1:4:1 (Davies and Law, 1983). All are phosphorylated to various extents, have an average monomer molecular weight of 23,300, and were

considered to have few specific secondary structural features, such as sheets or helices (Farrell, 1988). Recent infrared and Raman spectroscopic data, however, have demonstrated the existence of turns and more β -sheet than expected in casein monomers and polymers (Byler and Farrell, 1989; Byler *et al.*, 1988). The isolated fractions exhibit varying degrees and mechanisms of self-association that are thought to be mostly hydrophobically driven (Farrell, 1988; Holt, 1992; Schmidt, 1982). However, less work has been done on the tertiary and quaternary structures of these proteins in mixed associations in their native state. There is hydrodynamic evidence that, in the absence of calcium, whole casein associates to form aggregates with an apparent upper limit of 94 Å for the Stokes radius with a molecular weight of 220,000 (submicellar form) (Pepper, 1972; Pepper and Farrell, 1982), and this is in general agreement with

¹ Eastern Regional Research Center, ARS, USDA, Philadelphia, Pennsylvania 19118.

² To whom correspondence should be addressed. Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

the type of protein particles formed upon dissociation of casein micelles with chelating agents (Farrell, 1988; Holt, 1992; Schmidt, 1982).

It has long been hypothesized that *in vivo* upon the addition of calcium, these primarily hydrophobically stabilized, self-associated casein submicelles visible in electron micrographs of mammary tissue further aggregate via calcium-protein side-chain salt bridges to the colloidal micelles, with a size distribution centering upon 1500 Å diameter (Farrell, 1988; Holt, 1992; Schmidt, 1982). However, the exact supramolecular structures of the casein submicelles and micelles remain unknown. Models presented have ranged from those having discrete submicelles to those having the structure of a loose porous gel (Farrell, 1988), and to a newer model of a homogeneous sphere with a "hairy" outer layer (Walstra, 1990). For a recent review of casein micelle structure, the reader is referred to Holt (1992).

Recently, three dimensional models refined via energy minimization techniques were constructed for κ -casein (Kumosinski *et al.*, 1993b), α_{s1} -casein (Kumosinski *et al.*, 1994a), and β -casein (Kumosinski *et al.*, 1993a). These predicted structures were built from secondary structure sequence-based prediction algorithms in conjunction with global secondary structure results obtained from vibrational spectroscopy experiments (Byler and Farrell, 1989; Byler *et al.*, 1988). All energy-minimized structures were in good agreement with these global secondary structure determinations (Kumosinski *et al.*, 1993a,b, 1994a). Several energy-minimized aggregate structures were also presented to mimic the self-association processes for each of these caseins. In addition, qualitative speculation was presented for the interaction sites for κ -casein with α_{s1} -casein, but none were immediately obvious for κ -casein interaction sites with β -casein (Kumosinski *et al.*, 1993a,b).

In this paper, we have built an energy-minimized submicelle structure composed of one κ -casein with four α_{s1} -caseins and four β -caseins via plausible docking sites consistent with solution physical chemical, biochemical, and chemical experimental information. The energetics of the structures will be presented and the predicted structures will then be qualitatively compared with experimental structural and biochemical data, as well as geometric parameters calculated from the small-angle X-ray scattering results for submicellar particles.

2. METHODS

2.1. Molecular Modeling of Individual Caseins

The original monomeric casein models were constructed using a combination of sequence-based secondary structure prediction algorithms, constrained to be in compliance with experimental global secondary structure determined from vibrational spectroscopy (Kumosinski *et al.*, 1993a,b, 1994a). Since secondary structure sequence-based prediction algorithms are not informative with respect to turn type, individual caseins were reconstructed with differing turn types and these were tested by energy minimization. The model with the lowest energy was chosen. Thus, by this method a larger sampling of conformational space was performed. However, this methodology does not in any way allow one to conclude that a global energy minimization structure was achieved. This procedure is an extension of the method of Cohen and Kuntz (1989), where sequence-based secondary structure prediction methods are used as a starting point for tertiary structure prediction. Since the choice of the sequence-based algorithm is arbitrary (dubious), an added constraint of agreement between the individual or consensus algorithm and experimental global secondary results in conjunction with energy minimization techniques was utilized for sampling more of the possible conformational space. The resulting predicted energy-minimized models of κ -casein B, α_{s1} -casein B, and β -casein A are shown in Fig. 1A–C, respectively.

This methodology was tested with a polypeptide whose global secondary structure and X-ray crystallographic structure are known. In addition, the protein, like casein, should contain no disulfide bonds. Avian pancreatic polypeptide with 63 residues was chosen. Circular dichroism (CD) experiments of Noelken *et al.* (1980) showed a minimum of 80% helix under a variety of environmental conditions and the consensus prediction algorithm showed a polyproline helix for residues 1–9, α -helix for residues 13–32, and turns for residues 10 and 11. All other residues were given an extended conformation. After several energy minimization processes during which the type of turn was changed, the model with lowest energy, i.e., -599.4 kcal/mol, was chosen and is shown as a backbone-ribboned structure in the lower portion of Fig. 1D. The upper portion shows the backbone of the X-ray crystal structure (1PPT)

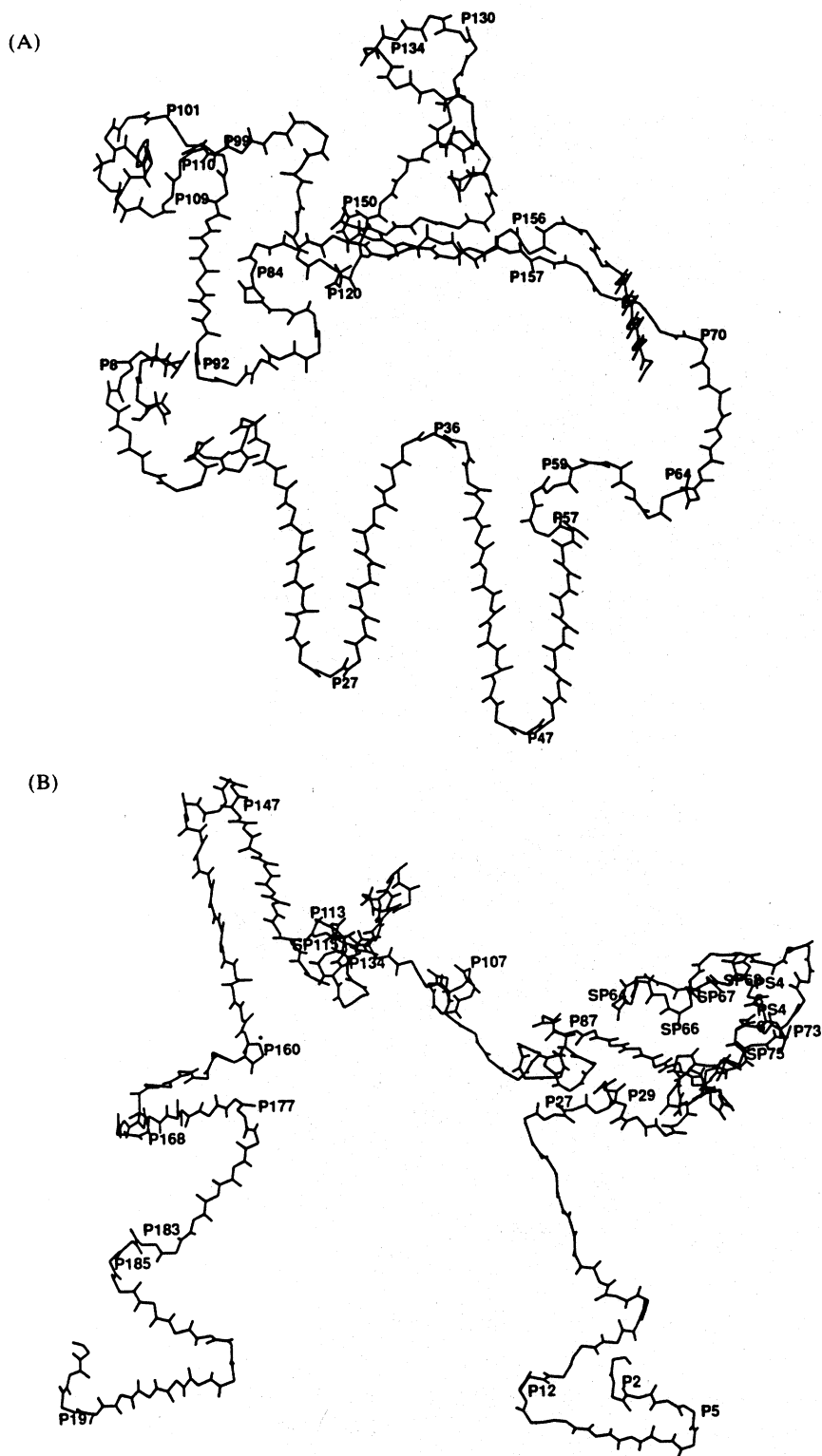
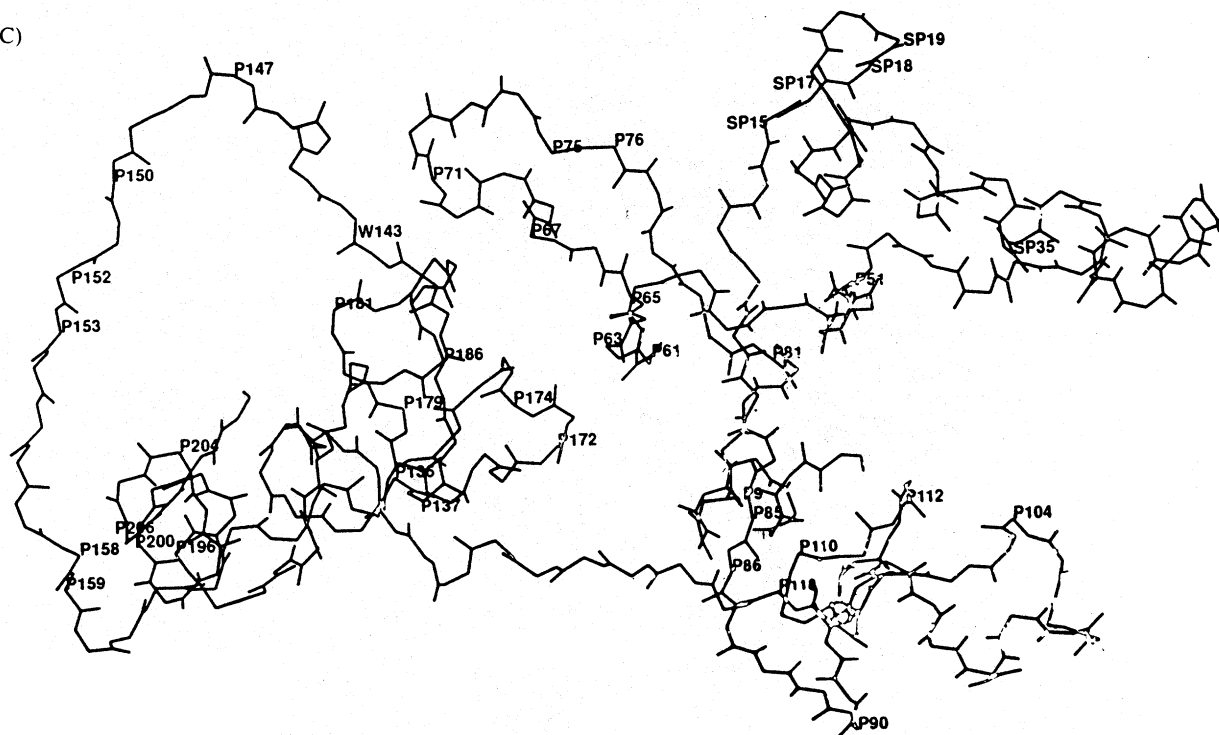


Fig. 1. (A) Backbone structure with labeled prolines (P) of κ -casein B. (B) Backbone structure with labeled prolines (P) and phosphoserines (SP) of β -casein A². (D) Ribboned backbone structure of avian pancreatic polypeptide: upper, X-ray structure (1 Ppt), lower, predicted energy-minimized structure

(C)



(D)

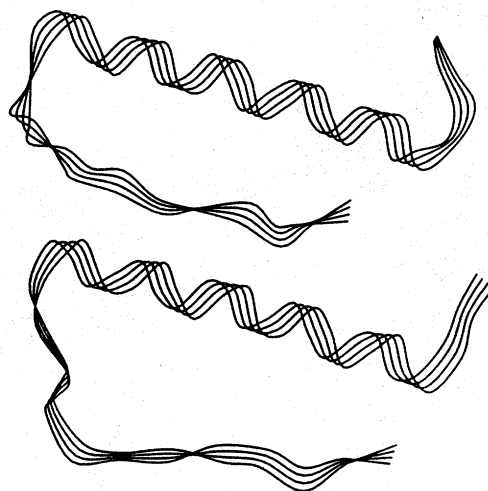


Fig. 1. Continued.

in this representation. Comparison of the backbone atoms between the predicted model and the X-ray structure yielded a root mean square deviation of 3.37 Å using the algorithm Fit of the Sybyl program. Hence, a reasonable low-resolution structure can be obtained by this methodology. It should be stressed that all structures built by these methods should be viewed as low-resolution working models and not

perfect structures for which the global energy minima have been attained.

2.2. Construction of Aggregate Structures

All complex aggregate structures employed the various casein monomer structures previously refined via energy minimization (Kumosinski *et al.*,

1993a,b, 1994a). Aggregates were constructed using a docking procedure on an Evans and Sutherland (St. Louis, MO) PS390 interactive computer graphics display driven by Sybyl molecular modeling software (Tripos, St. Louis, MO) on a Silicon Graphics (Mountainview, CA) W-4D35 processor. The docking procedure of this system allows the positions and orientations of up to four molecular entities to be independently manipulated. The desired orientations could then be frozen in space and merged into one entity for further energy minimization calculations utilizing a molecular force field. The criterion for acceptance of reasonable structures was determined by a combination of experimentally determined information and the calculation of the lowest energy for that structure. At least ten possible docking orientations were constructed, energy-minimized, and assessed for the lowest energy to provide a reasonable sampling of conformation space.

2.3. Molecular Force Field Energy Minimization

Empirical potential energy functions, although relatively crude, have been applied successfully to the study of hydrocarbons, oligonucleotides, peptides, and amino acids, as well as systems containing a large number of small molecules such as water. The AMBER force field (Weiner *et al.*, 1984, 1986) in Tripos' Sybyl software package was used in this study along with a 10–12 H-bond function. The parameters used for electrostatic calculations include atomic partial charges (q_i) obtained by the Kollman group (Weiner *et al.*, 1984, 1986) using a united atom approach with only essential hydrogens. All molecular structures were refined with an energy minimization procedure using a conjugate gradient algorithm, in which the positions of the atoms are adjusted iteratively so as to achieve a minimum potential energy value. Energy minimization calculations were terminated when the energy difference between the current and previous iterations was less than 1 kcal/mol of protein. A nonbonded cutoff of 5 Å was used initially to save computer time, and then an 8-Å cutoff was used as the structures became more refined. A stabilization energy of at least –10 kcal/mol residue of protein was achieved for all structures, which is consistent with values obtained for energy-minimized structures determined by X-ray crystallography.

3. RESULTS AND DISCUSSION

3.1. Casein Complex Structures

3.1.1. Synthetic Submicelle: Framework

Following the discovery by Waugh and von Hippel (1956) of κ -casein, the stabilizing factor of casein micelles, many studies aimed at understanding the nature of the protein–protein interactions involved were conducted. For the bovine system, these studies focused upon reconstituting micelles with mixtures of α_{s1} - and κ -caseins (Noble and Waugh, 1965; Schmidt and Payens, 1976). The reasons for this selection includes:

1. Synthetic micelles roughly resembling those of parent micelles could be formed from these two fractions alone.
2. Historically, β -casein was readily separable from the other fractions by mild procedures, so that it was not considered a primary reactant.
3. Separation of κ -casein from the α -complex had been a relatively difficult task, indicating a high degree of interaction.

All of these factors pointed toward the importance of α_{s1} -casein– κ -casein interactions in the bovine casein system (Kumosinski and Farrell, 1991; Noble and Waugh, 1965; Schmidt and Payens, 1976).

Initial micelle reconstitution experiments (Waugh and von Hippel, 1956) suggested that maximum stability of reformed micelles occurred at a ratio of 4:1 α_{s1} : κ -casein. Later, Noble and Waugh (1965) suggested a ratio of 10:1 overall but with stronger 1:1 complexes as nucleating sites. In consideration of these studies three factors are important: first, these were whole κ -casein fractions and Groves *et al.* (1992) have recently shown that these preparations contain polymers ranging up to octamers and above as well as some monomers, depending upon the degree of disulfide bonding; second, the ratio of 4:1:4:1 for α_{s1} : α_{s2} : β : κ -casein is about 9:1 in terms of phosphorylated calcium-sensitive caseins to κ -casein; finally, the redox potential of the bovine mammary gland lies far toward the reducing end of the scale, as the ratio of NADP to NADPH is 4×10^{-5} (Baldwin and Yang, 1974). All of these factors considered, along with the potential reactivity of κ -casein as a monomer (Woychik *et al.*, 1966), a ratio of 4:1 for the interaction of α_{s1} -casein with a reduced κ -casein

monomer appeared to be a logical starting point for the construction of a theoretical submicelle.

Figures 1A–C show the backbone structures for the energy-minimized models of κ -casein B, α_{s1} -casein B, and β -casein A², respectively. The κ -casein structure, which has been colloquially referred to as a “horse-and-rider” model (Fig. 1A), contains two sets of “dog-leg” structures. These so-called “dog-leg” structures are the result of two sets of antiparallel sheet structures each connected via a proline residue in a γ -turn configuration, i.e., prolines 27 and 47. It may be noted that for κ -casein, these prolines and the preceding and following sequences appear to be functionally preserved across a variety of species whose primary structures are known (Holt and Sawyer, 1993; Kumosinski *et al.*, 1993b). Hydrophobic groups, notably tyrosine and valine, which are evolutionarily conserved, are the predominant side chains located on both the smaller “dog-leg” (residues 20–34) and the larger one (residues 39–55). In addition, each “dog-leg” contains a lysine side chain near the pivotal proline residue; there is conservation of these positive charges in almost all κ -caseins (Holt and Sawyer, 1993; Kumosinski *et al.*, 1993b). This positive charge could conceivably form hydrophobically stabilized ion pairs with another “dog-leg” structure from a second casein containing an acidic group in a comparable position. Such “dog-leg” structures could easily be docked in an antiparallel fashion to maximize attractive dipole–dipole interactions and yield an acceptable stabilization energy. Candidates for this type of interaction can be found in the hydrophobic domain (left side of Fig. 1B) of the α_{s1} -casein B structure, which has two “dog-leg” structures, i.e., a large one (residues 136–159) and a smaller one (residues 162–175). Both of these “dog-leg” structures contain proline residues, i.e., residues 147 and 168, as pivotal points for the stranded antiparallel sheet structures. Unlike the γ -turn structures in κ -casein, these prolines are in the 2 position of a β -turn configuration, allowing for greater intrachain hydrogen bonding. The larger structure (residues 136–159 with a pivotal proline at residue 147) appears to have been deleted in ovine α_{s1} -casein, but is functionally conserved in rat at $n + 3$ residues from bovine (Holt and Sawyer, 1993; Kumosinski *et al.*, 1994a). The larger “dog-leg” thus has some variance in charge and size across species. In contrast, the smaller “dog-leg” centering on proline 168 is functionally preserved in all

species of α_{s1} -casein molecules examined, as is the proline at 160 which begins this structure (Holt and Sawyer, 1993; Kumosinski *et al.*, 1994a). Additionally, tryptophan 164 and tyrosine 166 are invariant and may potentiate hydrophobic interactions. There are no positive charges preceding the pivotal proline, but a negatively charged aspartic 175 is conserved following this residue (Holt and Sawyer, 1993; Kumosinski *et al.*, 1994a).

In a previous report, it was shown that the large “dog-leg” structure (residues 136–159) of α_{s1} -casein B was an excellent site of dimerization of α_{s1} -casein, yielding an interaction energy of -505 kcal/mole (-2113 kJ/mol) as calculated from the resulting difference between the energy of the dimer (column 3 of Table I) and two times the energy of the monomer (column 2 of Table I). Energy minimization of several other choices of sheet–sheet sites for α_{s1} -dimers always yielded 50–100 kcal/mol higher energies than the proposed model. The smaller “dog-legs” are conserved across species and thus preserve the suggested function, i.e., of interactions with κ -casein, the key to micelle structure for bovine caseins. Dimer formation (see Fig. 2A) at the larger “dog-leg” permits the easy docking of one of the small α_{s1} -casein “dog-leg” structures (residues 162–175) in an antiparallel fashion to a κ -casein “dog-leg” structure. In fact, the last residue of the small α_{s1} -casein “dog-leg” (aspartic 175) may interact with either lysine (24 or 46) on each of the “dog-leg” structures of κ -casein, resulting in a hydrophobically stabilized ion pair formation.

The choice of α_{s1} -casein interacting with κ -casein as a dimer is supported by the studies of van de Vroot *et al.* (1979), who examined the

Table I. Energy (in kcal/mol) of α_{s1} -Casein and β -Casein

Structure	α_{s1} -Casein		β -Casein dimer
	Monomer	Dimer	
Bond stretching	34.3	75.7	82.5
Angle bending	425.5	892.9	1,314.5
Torsional	427.3	840.9	44.6
Out-of-plane bending	15.6	36.6	602.6
1–4 Van der Waals	305.9	616.3	1,091.6
Van der Waals	–872.4	–1,805.0	–2,094.2
1–4 Electrostatic	2,135.3	4,268.2	4,400.1
Electrostatic	–4,426.3	–9,337.6	–10,230.0
H bond	–46.7	–96.4	–123.8
Total	–2,001.6	–4,508.5	–4,912.1

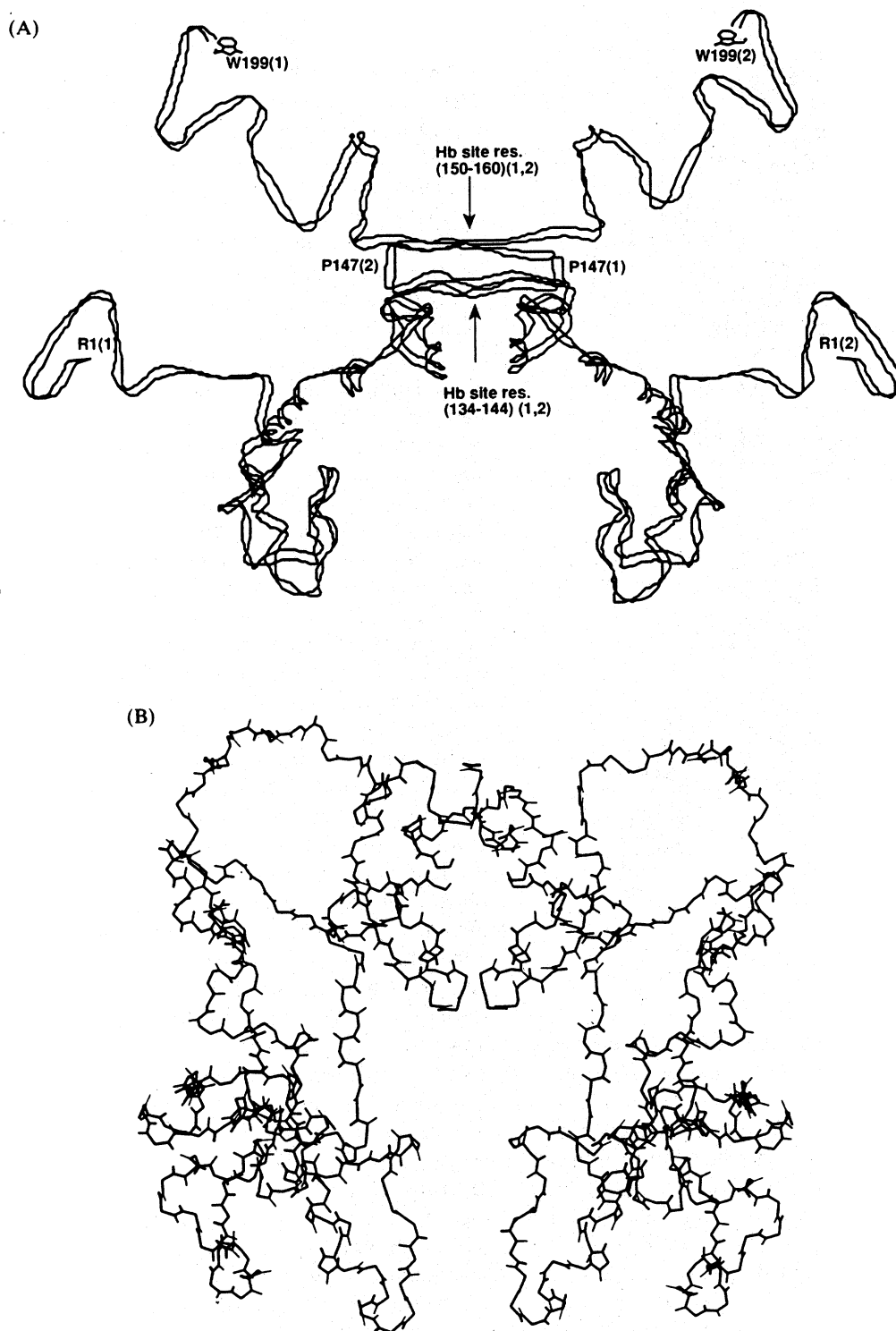


Fig. 2. (A) Double ribbon structure of α_{s1} -casein B dimer constructed by docking two large hydrophobic sheets in an antiparallel fashion; interaction sites noted (1, 2 refer to molecules 1 and 2). Hb, Hydrophobic site; W199, C-terminal tryptophan; R, N-terminal arginine; P147, proline 147. (B) Backbone structure for β -casein A² asymmetric dimer. (C) Backbone structure for β -casein A² symmetric dimer.

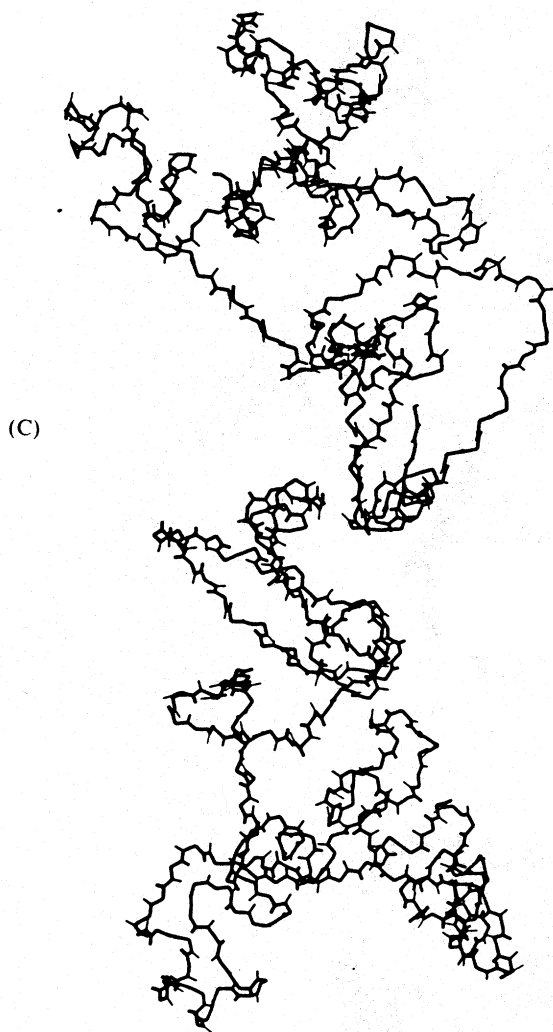


Fig. 2. Continued.

interactions of whole κ -casein and α_{s1} -casein by sedimentation equilibrium. They suggested dimer formation by α_{s1} -casein precedes α_{s1} - κ interactions. Furthermore, they demonstrated that κ - κ polymers were larger in size than the resultant α_{s1} - κ complexes, indicating a more energetically favorable state for the complexes. Pepper (1972) and Pepper and Farrell (1982) showed similar changes by analytical ultracentrifugation and by gel chromatography. Slattery and Evard (1973) observed complex formation between reduced κ -casein and α_{s1} -casein by sedimentation velocity studies. Association constants at 20°C for α_{s1} - and κ -casein complexes range from 2 to $8 \times 10^4 \text{ M}^{-1}$ ($K_D = 12\text{--}50 \text{ }\mu\text{M}$), depending upon the method of measurement (van de Vroot *et al.*, 1979). For the

polymerization of reduced κ -casein to its "micellar" complex an association constant of $4.5 \times 10^4 \text{ M}^{-1}$ ($K_D = 22 \text{ }\mu\text{M}$) can be calculated from Vreeman *et al.* (1981). Association constants for α_{s1} -casein polymerization range from 8 to $11 \times 10^4 \text{ M}^{-1}$ ($K_D = 9\text{--}12 \text{ }\mu\text{M}$) as calculated from Schmidt and Payens (1976) at 21°C and ionic strength equal to 0.1. Thus, considering the evidence for complex formation and the similarity of the association constants, here is a very high probability that α_{s1} and κ -caseins could form these postulated complexes.

Docking two α_{s1} -casein dimer structures via their small "dog-leg" structures in an antiparallel fashion with the two "dog-leg" structures of κ -casein, one interaction in front of the κ -casein and one behind the other "dog-leg" structure, yielded a rather spherically symmetric structure. Energy minimization of this model of one κ - with four α_{s1} -casein monomers yielded an excellent energy of $-11,811.1 \text{ kcal/mol}$ as seen in column 2 of Table II. The architecture of this refined synthetic submicelle structure is presented in Fig. 3A as a colored ribboned backbone structure in a relaxed stereo view. Figure 3B shows the complete structure with colored side chains, i.e., green hydrophobic, purple basic, red acidic, sulfur-containing orange, and all others cyan, and a ribbon structure for the backbone with the same color code as in Fig. 3A. Here, all the hydrophilic domains of α_{s1} -casein which contain the serine phosphates and acidic groups are located on the outside of the structure for easy access by water and calcium as potential sites for calcium binding and cross-linking, which leads to colloidal micelle formation. The dimeric phosphate clusters are, however, nearly diagonal (left to right) from each other, decreasing charge repulsions from the exterior phosphoserine clusters. The internal portion of this structure is divided into four open sectors (Fig. 3C). The two parallel to the κ -casein central structure (top and bottom of the top view of Fig. 3C) are largely hydrophobic in which fats and other hydrophobic solutes could bind. The other two quarters, which are perpendicular to the κ -casein (left and right of Fig. 3C), are hydrophilic and are easily water- and enzyme-accessible. The massive hydrophobic surface area produced by the two hydrophobic quadrants (Fig. 3C) can also be potential interaction sites for four β -casein structures via hydrophobic interactions, provided that the area is large enough not to cause poor van

Table II. Energy (in kcal/mol) for Refined Casein Submicelle Structure

Structure	Synthetic ^a	Submicelle ^b	
		Asym	Sym
Bond stretching energy	177.7	208.7	363.2
Angle bending energy	2,280.1	4,909.0	3,430.1
Torsional energy	1,996.4	2,888.4	3,272.6
Out-of-plane bending energy	93.1	200.5	496.1
1-4 Van der Waals energy	1,311.5	2,697.3	2,939.1
Van der Waals energy	-4,003.7	-8,379.8	-8,200.5
1-4 Electrostatic energy	12,046.7	19,000.7	19,292.8
Electrostatic energy	-25,454.9	-42,813.0	-45,120.4
H-bond energy	-258.1	-513.9	-519.0
Total energy	-11,811.1	-21,802.2	-24,047.0

^a Synthetic submicelle structure of one κ -casein and two α_{s1} -casein B dimers.

^b Submicelles with one κ -casein, two α_{s1} -caseins, and two β -caseins; Asym and Sym refer to the orientations of β -casein dimers.

der Waals contacts. It should also be noted at this time that the docking of four α_{s1} -casein alternative dimers of the κ -casein structures could not be accomplished by use of any of their larger "dog-leg" structures without producing large positive destabilization energies, caused initially by poor van der Waals contacts and strong electrostatic repulsions from phosphoserines placed in too close proximity. This (Fig. 3A-C) is the best structure yielding the lowest energy as determined by up to 10-15 docking combinations.

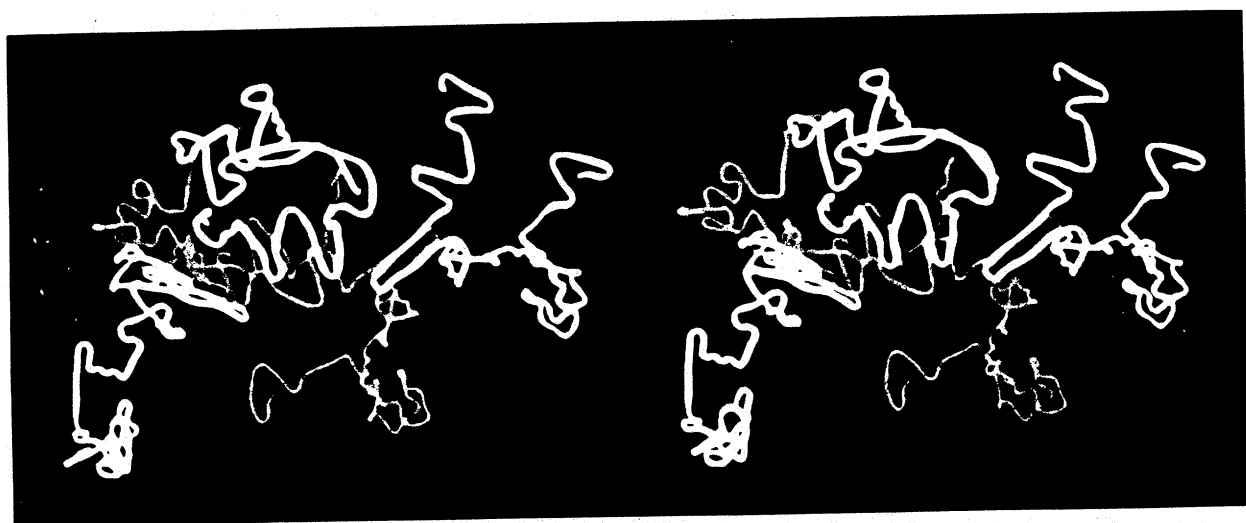
3.1.2. Submicelle Structure

As the interaction of β -casein with the micelle is regarded as primarily hydrophobic (Holt, 1992; Rose, 1968; Waugh *et al.*, 1970), it seemed plausible to dock two β -caseins within each of the two larger hydrophobic quadrants of the refined synthetic submicelle structure which occur to the right and left of the "rider" (see Fig. 3B, C). How this should be performed was not immediately evident.

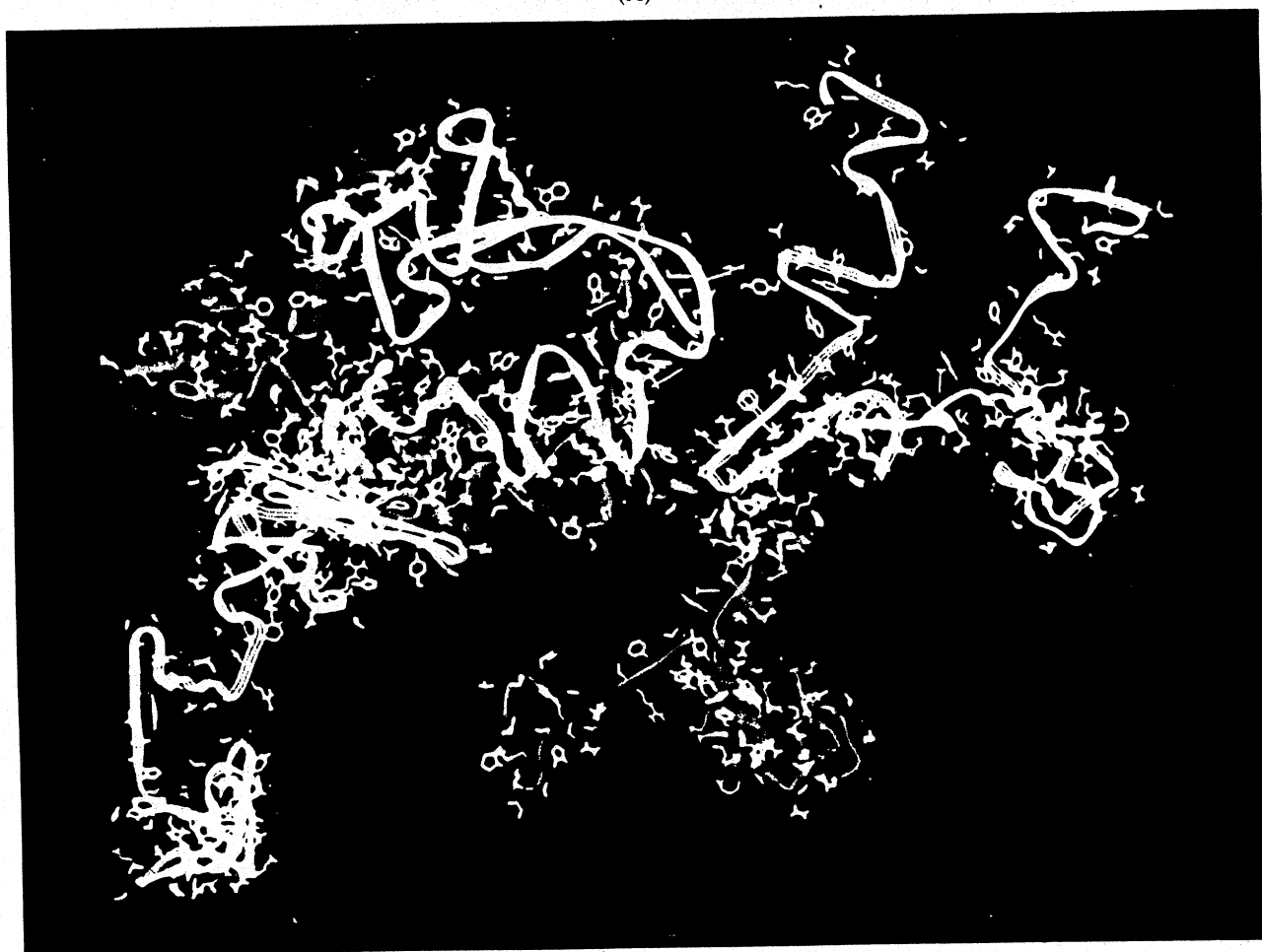
In a previous report, the energy-minimized structures of β -casein monomer and aggregates were presented (Kumosinski *et al.*, 1993a). The β -casein model resembles a detergent molecule inasmuch as one end of the molecule contains two hydrophilic arms while the other end contains predominantly hydrophobic side chains. To comply with chymosin cleavage experiments of Creamer (1976), an asymmetric dimer was constructed as a precursor to β -casein polymeric structures (Kumosinski *et al.*, 1993a). (The terms symmetric and

asymmetric will be used here to describe structures with and without a center of inversion, respectively.) In the resulting dimer, all the hydrophilic groups remain on one side of the structure and the hydrophobic groups are located on the other side (Fig. 2B). After energy minimization this dimeric structure yields a total energy of -4912.1 kcal/mol (column 4 of Table I). Two of these β -casein A² dimers were then docked in an asymmetric fashion within the two hydrophobic quadrants of the synthetic submicelle framework structure of Fig. 3B, with their hydrophilic arms pointed outward from the central cavity. This resulting structure (Fig. 4A, B) was next energy-minimized and yielded an acceptable energy of over -10 kcal/mol/residue (Table II). Although no stabilization energy is observed, such a structure is highly likely, since the interaction between the β -casein dimers and the synthetic submicelle structure is for the most part hydrophobic, which is supported by the dissociation of β -casein from submicelles and micelles at 4°C and below (Ali *et al.*, 1980; Davies and Law, 1983; Downey and Murphy, 1970; Rose, 1968).

Another submicellar structure, which will be referred to as a symmetric model, can be built by the symmetric docking of two β -casein A² symmetric dimers into the two hydrophobic cavities of the synthetic submicelle structure. The β -casein A² symmetric dimer (Fig. 2C) contains two hydrophilic sites at either end of the structure and a central hydrophobic region. The symmetric dimer has no loss in stabilization energy and after



(A)



(B)

Fig. 3. Refined structure of casein synthetic submicelle framework, i.e., one κ -casein B and four α_{s1} -casein B monomers. (A) Stereo view (relaxed) of ribboned backbone without side chains. Color code: κ -casein with cyan ribbon and α_{s1} -casein with red and green ribbons. (B) Same view as in (A) with side chains: ribbon backbone color same as in (A); side chains are green for hydrophobic, red for acidic, and blue for basic groups. (C) Top view backbone atoms only, κ -casein cyan, two α_{s1} -casein molecules green, and one each red and magenta.

(C)

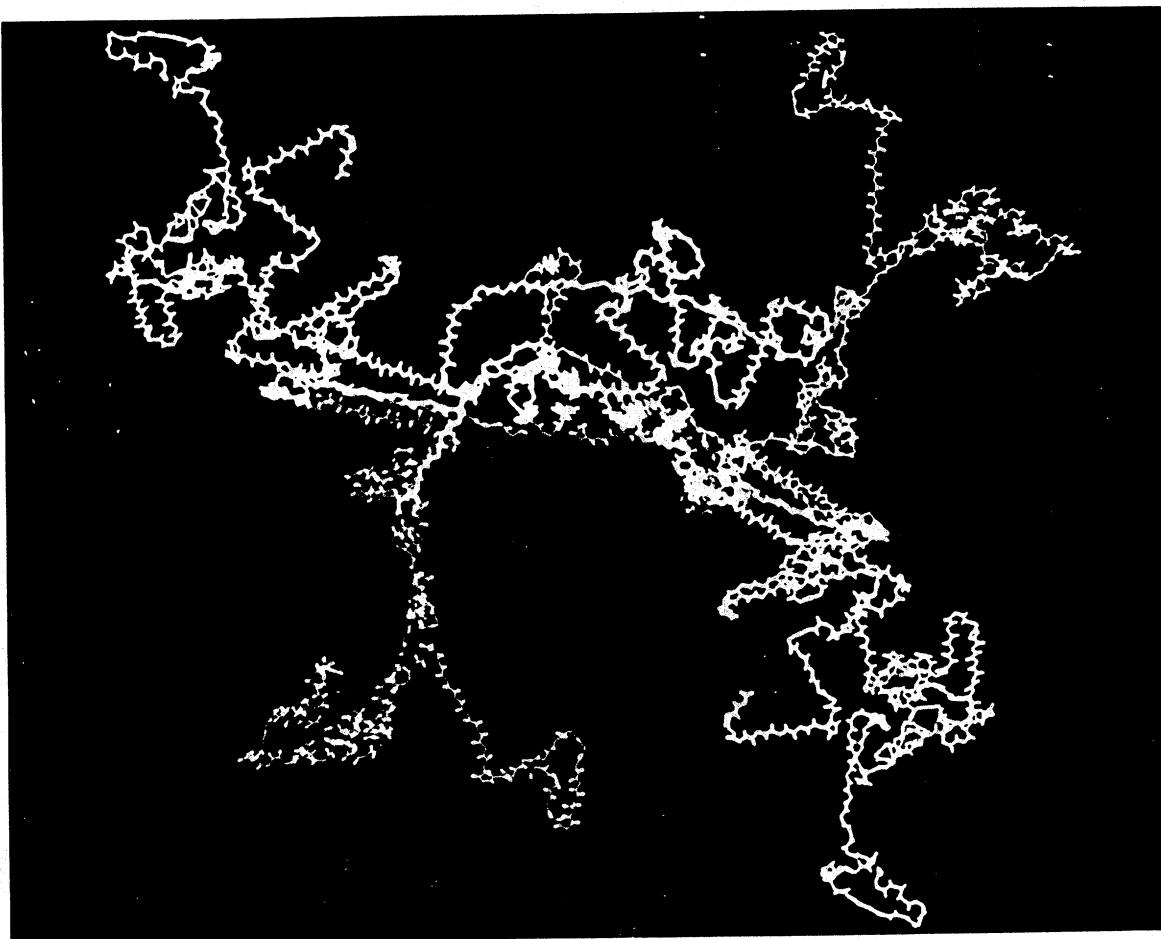


Fig. 3. Continued.

(A)

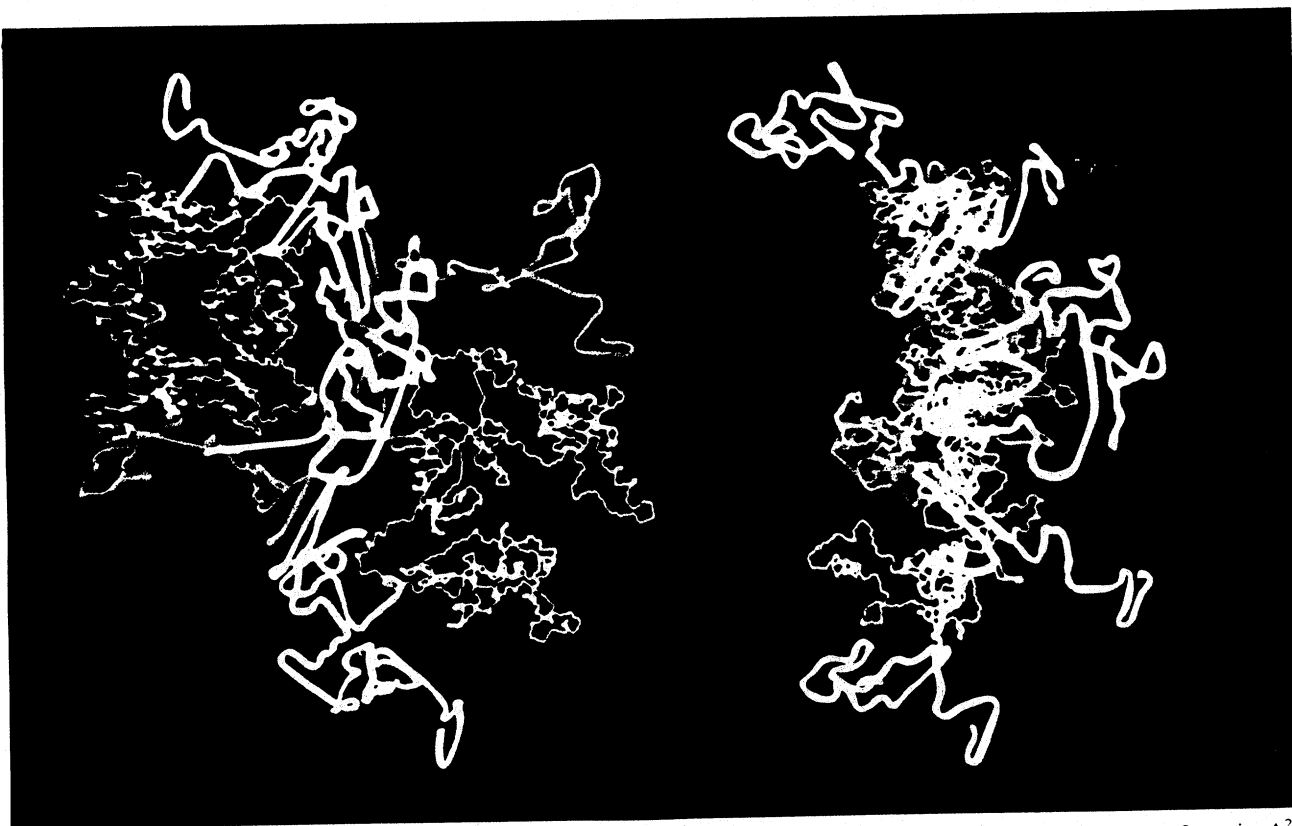


Fig. 4. Energy-minimized casein asymmetric submicelle structure, i.e., one κ -casein B, four α_{s1} -casein B, and two β -casein A² asymmetric dimers. (A) Orthogonal view of ribboned backbone without side chains: κ -casein B cyan, α_{s1} -casein B red and green; β -casein A² backbone without O and H atoms are magenta. (B) Stereo view (relaxed) of (A) structure.

(B)

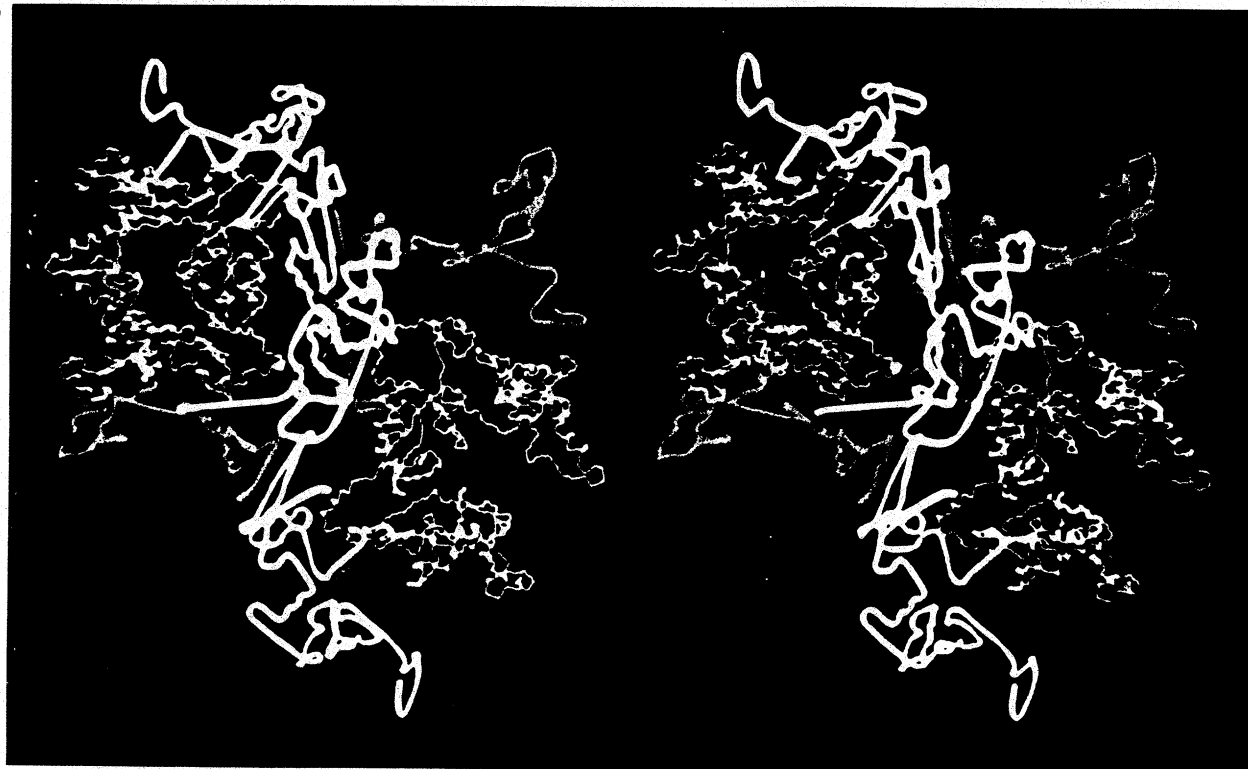


Fig. 4. Continued.

minimization gives a total energy of -5484.4 kcal/mol, which is different from the asymmetric dimer and will be discussed in detail in Section 3.2. In this submicelle structure, the symmetric dimers must be docked with their central hydrophobic portion in contact with the hydrophobic cavity of the synthetic submicelle structure so that their

hydrophilic areas are actually perpendicular to corresponding β -casein dimeric structures docked within the asymmetric submicellar model (Fig. 4A, B). The resulting structure was energy-minimized and is presented in Fig. 5A, B. It should be noted that one of the β -casein hydrophilic portions of the symmetric dimer partially covers the view of the

(A)

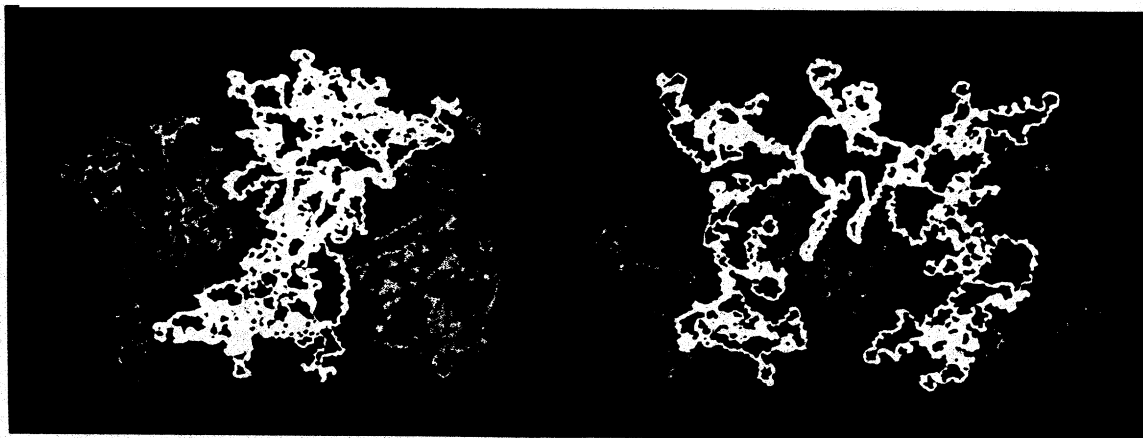


Fig. 5. Energy-minimized casein symmetric submicelle structure, i.e., one κ -casein, four α_{s1} -casein B, and two β -casein symmetric dimers. (A) Orthogonal view of backbones without side chains; κ -casein B cyan, α_{s1} -casein B red, and β -casein A² magenta. (B) Stereo view (relaxed) of (A) structure.

(B)

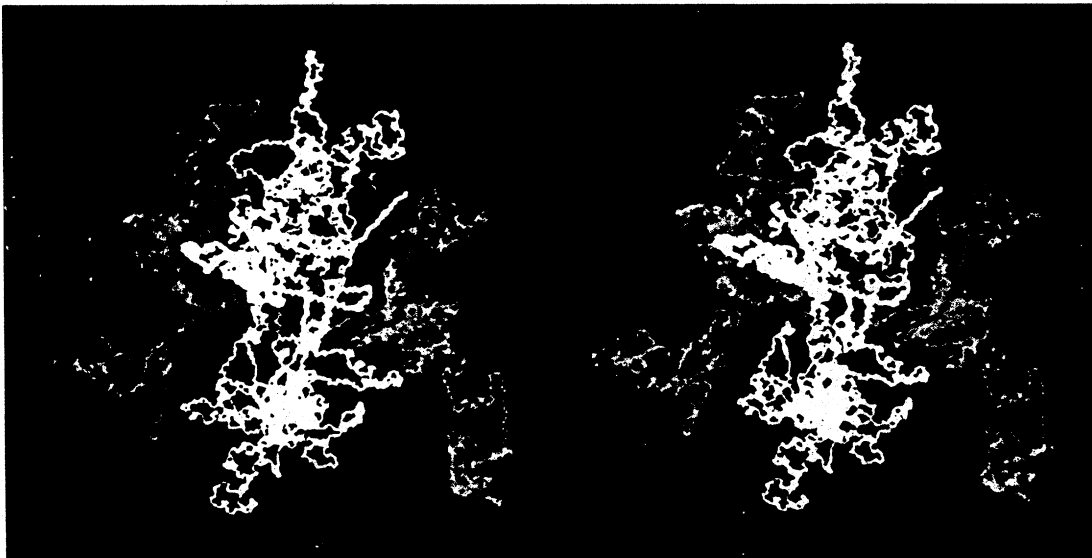


Fig. 5. Continued.

GMP of the κ -casein within this structure, but it in no way interferes with access of the chymosin to the phenylalanine-methionine cleavage site of κ -casein, nor does it hinder access to glycosylation sites on the κ -casein.

Several other approaches to docking β -casein

dimers were attempted. Most of these resulted in extreme loss of stabilization energy. The dominating factor in docking the four β -caseins is the proximity of the four hydrophilic ends to each other and to the phosphate-rich portions of α_{s1} -casein. Charge repulsions in these areas prevent many

(A)

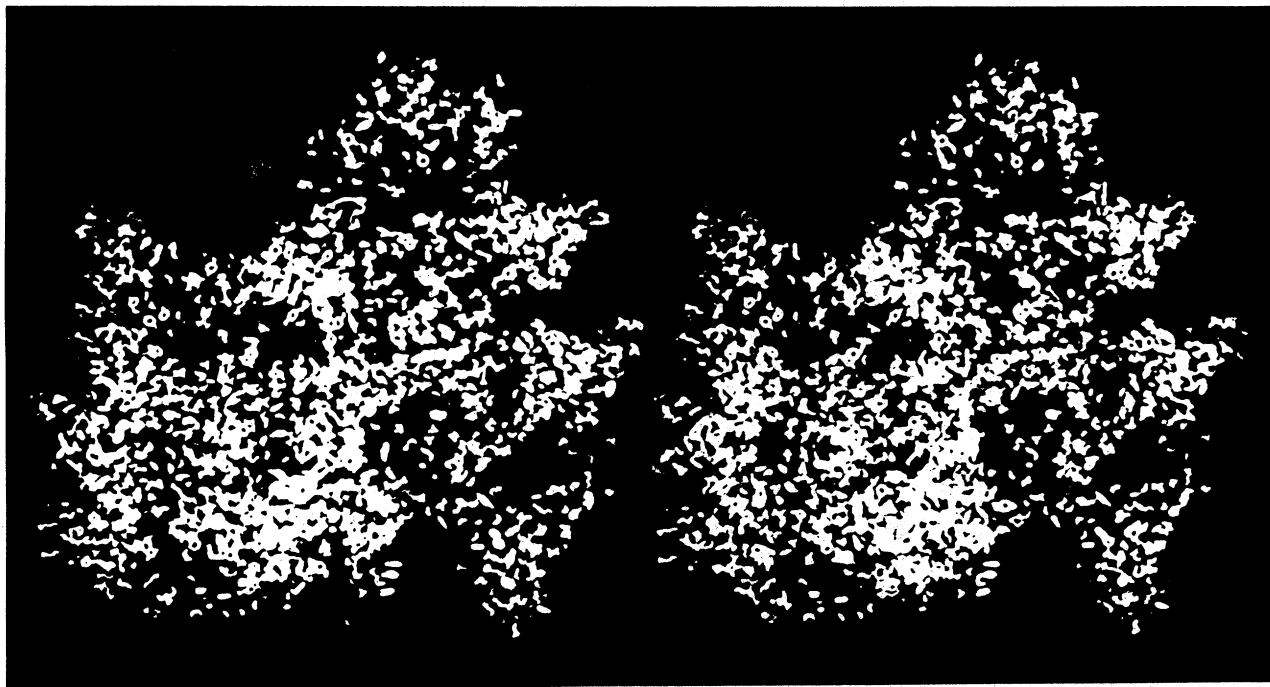


Fig. 6. Stereo view of refined casein asymmetric submicelle structure with side chains and without backbone atoms. Same view as in Fig. 4B. Hydrophobic side chains green, acidic red, and basic purple, while S-S or S-H groups are orange. β -casein renin cleavage sites are orange, plasmin sites are red-orange.

hypothetical approaches. To add validity to the above refined structures, which are energy-minimized, comparisons of the models with their energetics as well as with experimental evidence derived from solution studies such as Raman spectroscopy, chemical and biochemical results, and solution physicochemical studies will be presented.

3.2. Energetics of Casein Submicelle Formation

In the preceding sections, we have speculated that a synthetic submicellar structure consisting of one κ -casein B and four α_{s1} -casein B monomers (seen in Fig. 3A–C) may exist prior to the formation of the total submicellar structure resulting from the further interaction of two antiparallel β -casein dimers with the submicellar framework. To test such a mechanism, we now utilize the total energy calculations of each structure. For these calculations, we rely on the fundamental principle that molecular structure should occur primarily in their lowest energy state. Thus, the structure with the lowest energy has the highest probability of existence in solution, providing it is not in a local minimum. It must also be remembered that these calculations are for static structures carried out in the absence of water; addition of water will be discussed in a subsequent paper.

3.2.1. Synthetic Submicelle

As seen in column 2 of Table II, the total energy or internal energy of the synthetic submicelle framework (Fig. 3A, B) is $-11,811.1$ kcal/mol. From this total energy, the monomer energy of κ -casein, i.e., -2134.3 kcal/mol (column 2 of Table I of Kumosinski *et al.*, 1993b), and the dimer energy of α_{s1} -casein B (column 3 of Table I), it is possible to calculate a stabilization or formation energy due to the interaction of κ -casein with the small “dog-legs” of the α_{s1} -dimer. The calculated value is -659.8 kcal/mol (-2760 kJ/mol), i.e., $-11,811.1 - [-2134.3 - 2(4508.5)]$; it represents 5.6% of the total energy and is well above the 1% limit of acceptability we imposed. This stabilization energy is primarily due to the antiparallel docking of the κ -casein “dog-leg” with the small “dog-leg” of the α_{s1} -casein B dimer resulting in a more favorable dipole–dipole interaction. Closer inspection of the force field terms responsible for this stabilization energy indicates that 75% of this energy is due to

electrostatic interactions, while 25% is due to van der Waals interactions. Hence, electrostatic interactions (the dipole–dipole interactions of the backbone “dog-legs”) are the predominant forces responsible for the formation of a synthetic submicelle structure. Note also the importance of the species-conserved positive charges of κ -casein (residues 24 and 46) and the conserved negative charge of α_{s1} -casein (residue 175). The 25% contribution of the van der Waals energy is probably the result of other side chain–side chain interactions. No excess hydrogen bonding was evident. Quite simply, a configurational internal energy due to electrostatics is also responsible for stabilizing a synthetic submicelle structure in addition to the known hydrophobic side-chain interaction. In real solutions, the κ -casein as well as the α_{s1} -casein will tend to self-associate, but as noted above, experimental evidence has been presented to support the formation of α_{s1} -casein– κ -casein complexes with association constants of the same order of magnitude as those for self-association. The question to be addressed for this model is whether the calculated κ – α_{s1} “dog-leg” stabilization energy is lower in magnitude than the κ – κ “dog-leg” interaction energy, because, as previously shown (Kumosinski *et al.*, 1994a), models for the self-association of α_{s1} -casein to larger aggregates do not involve the small “dog-leg” antiparallel sheet structures used for docking with the κ -casein in this study. In a previous report (Kumosinski *et al.*, 1993b) association energy of the dimer of κ -casein formed by the antiparallel interaction of its two smaller “dog-leg” sheets was found to be -423.8 kcal/mol (-1773 kJ/mol). The κ -tetramer is formed by the interaction of two dimers and the association energy of the tetramer minus two times the dimer association $-920.2 - 2(-423.8)$ is -72.6 kcal/mole (-304 kJ/mol). Therefore, the total self-association energy of a κ – κ small and large “dog-leg” interaction is -496.4 kcal/mol (-2077 kJ/mol), i.e., the sum of -423.8 and -72.6 kcal/mol. Now, as previously discussed in this section, the interaction energy of κ -casein “dog-legs” with two small “dog-leg” α_{s1} -casein B dimers is equal to -659.8 kcal/mol. Hence, the interaction of κ -casein with α_{s1} -casein by the mechanism presented will be energetically favorable over the κ – κ -casein self-association by -163.4 kcal/mol (-684.7 kJ/mol), i.e., $-659.8 - (-496.4)$ kcal/mol.

In addition to electrostatic and van der Waals

interactions, it would be advantageous at this time to test the experimentally verified hypothesis (van de Vroot *et al.*, 1979) that hydrophobic interactions are also responsible for synthetic submicelle formation. For this calculation, we shall use the hydropathy scale of Kyte and Doolittle (1982), noting that the "dog-legs" of both α_{s1} -casein and κ -casein contain hydrophilic as well as hydrophobic side chains. Inspection of Fig. 1A shows that best contacts for the small and large "dog-legs" of κ -casein are residues 21–33 and 41–52, respectively. Use of the hydropathy scale yields apparent energies -7.3 and -11.2 kcal/mol for the small and large "dog-leg" structures of κ -casein, respectively. It should be noted that for hydrophobic bond formation, hydrophobic groups yield negative free energies while hydrophilic groups are positive. Hence, the hydrophobic energy of formation of κ - κ "dog-leg" self-association is $(-7.3) + (-7.3) + (-11.2) + (-11.2) = -37.0$ kcal/mol. Other hydropathy scales (Edsall and McKenzie, 1983) yielded similar results.

Inspection of Fig. 1B and 2A shows that the residues of the small "dog-leg" of α_{s1} -casein, responsible for interaction with the "dog-legs" of κ -casein, are 162–175 centering on proline 168. These residues yield an energy of -14.9 kcal/mol on the hydropathy scale. The interaction energy for synthetic submicelle formation due to hydrophobic side-chain interactions can be calculated as $(-14.9) + (-7.3) + (-14.9) + (-11.2) = -48.3$ kcal/mol. Thus, hydrophobically driven side-chain interactions also participate in the formation of the synthetic submicelle framework structure over a κ -casein self-association by a stabilization energy of -11.3 kcal/mol (-47.3 kJ/mol), i.e., $(-48.3) - (-37)$ kcal/mol.

From the above structure and energetics requirements, it can be postulated that in a solution of κ -casein and α_{s1} -casein, a synthetic submicelle will form over κ -casein aggregates because of a decrease in the internal energy of -163.4 kcal/mol, 75% of which is electrostatic and 25% van der Waals, and a side-chain hydrophobic Gibbs free energy of -11.3 kcal/mol. However, these two calculated energies cannot be summed to yield a realistic thermodynamic value, because they are potential energies rather than free energies. Nevertheless, the calculation of both parameters yields a qualitative mechanism for a κ -casein and α_{s1} -casein synthetic submicelle formation which is in agreement with experimental evidence (Pepper,

1972; Pepper and Farrell, 1982; Slattery and Evarad, 1973; van der Vroot *et al.*, 1979).

3.2.2. Asymmetric Submicelle

The addition of two β -casein A^2 dimers to the synthetic submicelle framework does not yield any dramatic improvement in energetics as was the case for the synthetic submicelle intermediate structure. Here, we calculate for an asymmetric dimer, as seen in Fig. 2B, an energy of -4912.1 kcal/mol (Table II). From a previous report (Kumosinski *et al.*, 1993a), a monomeric energy value of -2742.2 kcal/mol ($-11,493$ kJ/mol) for β -casein A^2 was calculated using a Kollman force field. The formation of an asymmetric β -casein A^2 dimer (Fig. 2B) yields a destabilizing internal energy of 572.3 kcal/mol (2394.4 kJ/mol), i.e., $-4912.1 - 2(-2742.2)$ kcal/mol. Furthermore, the calculated internal energy of $-21,802.2$ kcal/mol (-9122 kJ/mol) for the submicelle structure (column 3 of Table II) is well within our self-imposed 1% limit from a value of $-21,635.3$ kcal/mol ($-90,552$ kJ/mol) calculated from the energy of the synthetic micelle and two asymmetric β -casein A^2 dimers. Thus, the addition of β -casein does not result in favorable electrostatic or van der Waals interactions for the asymmetric submicelle model. However, it is important to note that this interaction is not disallowed either, as was the case with several other structures where the configuration of the added β -casein yielded unfavorable electrostatic repulsions which dramatically increased the internal energy of the system. As previously shown (Kumosinski *et al.*, 1993a), the C-terminal half of β -casein A^2 is extremely hydrophobic with a low charge frequency and so it is reasonable to speculate that β -casein interacts with the α_{s1} -casein and κ -casein synthetic submicelle structure via side-chain hydrophobic interactions. The phenomenon of low-temperature dissociation of β -casein from complexes apparently occurs under a variety of conditions (Ali *et al.*, 1980; Davies and Law, 1983; Rose, 1968).

3.2.3. Symmetric Submicelle

The addition of two β -casein A^2 symmetric dimers to the synthetic submicelle yields better results than for the asymmetric submicelle structure. Here, we calculate for a symmetric dimer, as seen in Fig. 2C, which yields an energy of

−5484.4 kcal/mol. From a previous report (Kumosinski *et al.*, 1993a) a monomeric energy value of −2742.2 kcal/mol was calculated for a β -casein A² monomer. The formation of a symmetric β -casein A² dimer (Fig. 2C) yields no destabilizing internal energy, i.e., $-5484.4 - (2)(-2742.2) = 0$ kcal/mol. Furthermore, the calculated internal energy of −24,047.0 kcal/mol for the symmetric submicelle structure (column 4 of Table II) is 4% above the value of −22,779.9 kcal/mol calculated from the energy of the synthetic micelle and two symmetric β -casein A dimers. Hence, the addition of β -casein in a symmetric dimer results in a favorable electrostatic or van der Waals interaction, i.e., −1267.1 kcal/mol. Also, since the central portion of the symmetric β -casein A dimers are docked into the hydrophobic cavities of the synthetic submicelle, a large negative hydrophobic energy would be expected to occur to the same extent in this structure as in the asymmetric submicelle model. Here, not only do hydrophobic, electrostatic, and van der Waals interactions stabilize this symmetric structure, but also the resulting structure (Fig. 5) is more spherically symmetric with a dipole of 9078 Debye units more than the corresponding asymmetric model, $\mu = 12,312$. However, choosing one of the two structures over the other would be highly speculative and imprudent at this time. More comparisons of these structures with solution structural experimental studies must be initiated before such conclusions can be reached.

3.3. Comparison of the Predicted Models with Secondary Structural Analysis

Global secondary structural analysis showed little differences between the two models. Calculated results were compared with experimentally

obtained global secondary structure from FTIR and Raman spectroscopy (Table III). No significant changes in the ϕ , ψ angles of the backbone peptide bonds between the individually refined κ -, α_{s1} -, and β -casein structures and those within the two submicelle structures were observed. Table III also contains the secondary structure analysis calculated in previous communications for the individual casein structures. The results of this calculation given in Table III are in reasonable agreement with the experimentally determined values from Raman spectroscopy (row one of Table III), even though the Raman spectroscopy experiments were performed on a dry lyophilized powder of whole sodium caseinate. While this type of analysis does not prove the refined submicelle structure, it does add further support to the possibility of such a refined model.

2.4. Comparison of the Predicted Models with the Chemistry of Whole Casein

3.4.1. Chymosin (Rennin) Hydrolysis

The action of chymosin on the casein micelle is primarily the hydrolysis of the highly sensitive phenylalanine-methionine peptide bond (residues 105–106) of κ -casein. We have speculated (Kumosinski *et al.*, 1993b) that proline-rich areas preceding and following this bond cause the formation of a kink which presents the otherwise hydrophobic chymosin-sensitive bond on the surface of the monomer model of κ -casein. In both of the predicted submicelle models this segment remains on the surface and is therefore readily accessible to chymosin.

In α_{s1} -casein, the phenylalanine-phenylalanine bond (residues 23 and 24) is a major

Table III. Comparison of Initial Structures with Spectroscopic Data

Sample		% Helix	% β -Structure	% Turns	% Unspecified
Submicelle (lyophilized)	FTIR and Raman ^a	8–18	24–30	36–39	16–32
κ -Casein	Refined	16	27	30	26
α_{s1} -Casein	Refined	8	18	34	40
β -Casein	Refined	10	20	34	36
Submicelle ^b	Calculated	10	20	34	36

^a Byler and Farrell (1989), Byler *et al.* (1988).

^b Constructed for asymmetric and symmetric models (see text).

chymosin cleavage site (Fox, 1981; Mulvihill and Fox, 1979) and it is surface-accessible in both of the predicted submicelle models. Additional chymosin cleavage sites of α_{s1} -casein are located between residues 32–33, 149–150, 169–170, and 179–180 (Fox, 1981; Mulvihill and Fox, 1979; Visser, 1981). The site between residues 32 and 33 is relatively exposed in both predicted models, thus facilitating enzymatic attack. The last three sites, 149–150, 169–170, and 179–180 appear to be much less exposed and could be involved in hydrophobic sheet–sheet interactions to form α_{s1} -dimer (136–159) or act as κ - α_{s1} sites of interaction (162–175).

The chymosin cleavage sites of β -casein while being exposed on the surface of the monomer model are located on the hydrophobic (left) end of the proposed structure (see Fig. 1C). Hence incorporation of these sites into the micelle models would predict that chymosin action on β -casein would be inhibited in whole casein. In fact, Visser (1981) and Creamer (1976) have shown that successful chymosin action on β -casein sites occurs either at low temperatures where hydrophobically driven self-association is minimized or at low pH. Berry and Creamer (1975) successfully cleaved sites 189–190 on β -casein at 2°C and the resulting large peptide, i.e., residues 1–189, showed marked decrease in self-association by gel-permeation chromatography at elevated temperatures. In our structure, residues 189–190 are located in the interior hydrophobic cavities and are not easily accessible to enzyme action if hydrophobic self-association into submicelles has not occurred. This would be in accord with the low reactivity of chymosin toward β -casein in early stages of cheese making (Fox, 1981).

3.4.2. Hydrophobic Interactions

In the construction of the submicelle framework model, residues 35–68 which represent an exceptionally hydrophobic area with almost no charge (the “legged” structures of the κ -casein molecule) were used as sites for sheet–sheet interactions with the smaller hydrophobic “dog-legs” of α_{s1} -casein. Several investigators (Ali *et al.*, 1980; Davies and Law, 1983; Downey and Murphy, 1970; Rose, 1968) have noted that β -casein and to a certain extent κ -casein diffuse out of the casein micelle at low temperatures. As one decreases the temperature, hydrophobic stabilization energy decreases, and κ -casein and β -casein are more likely

to dissociate from the micelle, leaving behind a calcium-stabilized framework (Lin *et al.*, 1972). This is in accord with the overall hydrophobic nature presented here for β -casein interactions in the submicelle models. Finally, the importance of the hydrophobic region in α_{s1} -casein- κ -casein interactions can be supported by the research of Woychik and Wondolowski (1975). Of nine tyrosine residues in κ -casein, seven are located between residues 35 and 68; nitration of seven tyrosines in κ -casein severely inhibited its ability to stabilize α_{s1} -casein. As can be seen in Fig. 1A, the “legged” structures are constituted from this region and contain seven tyrosine residues; similar results were obtained upon nitration of the tyrosine residues of α_{s1} -casein (Woychik and Wondolowski, 1975).

3.4.3. Sulfhydryl–Disulfide Interactions

κ -Casein contains two cysteine residues (Eigel *et al.*, 1984). The occurrence of free sulhydryl groups in the milk–protein complex has been reported by Beeby (1964) but not by others (Swaigood *et al.*, 1964). Woychik *et al.* (1966), however, demonstrated that reduced and alkylated κ -casein stabilized α_{s1} -casein against calcium precipitation as well as native κ -casein. In the monomer and submicelle models cysteine residues (11 and 88) are located near the surface of the molecule and are directed away from each other at a distance of over 33 Å. This could account for the ability of the κ -casein molecule to form the interchain disulfide-bonded polymers between submicelles after secretion (Groves *et al.*, 1992). No disulfide bonding has been considered here because of the presumed reducing atmosphere of mammary gland (Baldwin and Yang, 1974).

3.4.4. Sites for Glycosylation and Phosphorylation

Of the major components of the casein complex, only κ -casein can be glycosylated. Nearly all of the carbohydrate as well as the phosphate associated with κ -casein is bound to the macropeptide (Eigel *et al.*, 1984), which is the highly soluble portion formed by chymosin hydrolysis. The major site for glycosylation, Thr-133, is found in our model on the back of the “rider” and is on a β -turn and is readily accessible to solvent. The sites of phosphorylation, Ser-149 and Thr-145, are also on the back portion and are found in β -turns as well.

The α_{s1} -casein B molecule contains eight

phosphate residues, all in the form of serine monophosphate (Eigel *et al.*, 1984). Seven of these phosphoserine residues are clustered in an acidic portion of the molecule bounded by residues 43 and 80 (the second fifth of the molecule from the amino-terminal end) and the protein is readily precipitated by Ca^{2+} (Farrell *et al.*, 1988). The monomer model shows seven of these phosphate residues to be located on β -turns (Fig. 1B), which is compatible with known phosphorylated residues in crystallized proteins (Kumosinski *et al.*, 1934a). Because of electrostatic calculations, these serine phosphates are predicted to be on the surface of the framework model and on the surface of both submicelle models. For β -casein four of its five phosphoserine residues (Fig. 1C) are located in an extended loop represented by the "head of the crab" in our colloquial description (Kumosinski *et al.*, 1993a). The fifth residue at position 35 is found in the upper hydrophilic arm (Fig. 1C). In the submicelle models all of these phosphoserine residues also protrude outward to the surface. In construction of the submicelle models, little emphasis was placed on casein phosphate groups as stabilizers of submicelle structure, as submicellar-like complexes can form in the absence of phosphate (Farrell, 1988). However, almost all phosphoserine residues are directed outward where they are accessible for enzymatic attack or for the Ca^{2+} binding necessary to form colloidal casein micelles.

3.4.5. Plasmin Cleavage Sites for β -Casein

The plasmin cleavage sites (Eigel *et al.*, 1984), residues 28–29, 105–106, and 107–108, are readily exposed in the β -casein monomer model as seen in Fig. 1C, and they are also readily exposed on the surface of the proposed submicelle structures. Hence, all sites are not only solvent-accessible and easily exposed to enzyme action, but would also predict that plasmin would most rapidly enzymatically attack these sites at all temperature and salt concentrations. This fact is indeed corroborated by many past studies (Eigel *et al.*, 1984) and recent work with trypsin (Leaver and Thomson, 1993).

3.4.6. Carboxypeptidase

An intriguing aspect of casein biochemistry is the finding that carboxypeptidase A can readily liberate the C-terminal groups of all caseins regardless of their degree of self-association or

incorporation into micelles (Ribadeau-Dumas and Garnier, 1970). In both predicted models, the C-terminals of the α_{s1} - and κ -caseins are accessible, those of β -casein less so, but the C-terminal valine is projected to the outside of the monomer and dimer structure by its β -spiral structure (Kumosinski *et al.*, 1993a). In the asymmetric model the β -casein C-terminals are partially buried, whereas in the symmetric model they appear predominantly exposed.

3.5. Comparison of the Predicted Models with Solution Structural Results

The energy-minimized, three-dimensional models of the casein asymmetric and symmetric submicelles, shown in Figs. 4A, B, 5A, B, 6, and 7, can all be approximated by a spherical particle with two electron densities. The distance from one end of the β -casein through the κ -casein structure and to the end of the opposite β -casein molecule is 100 Å in both models. The packing density of this region would be considered higher than any other within the structure (Figs. 6 and 7). The longest distance measured from one α_{s1} -casein hydrophilic domain to an opposite one is about 200 Å (Figs. 6 and 7). These values, symmetry, and packing densities agree qualitatively with recent small-angle X-ray scattering (SAXS) results (Farrell *et al.*, 1990; Pessen *et al.*, 1991). Here, the SAXS data for casein submicelles were modeled as an inhomogeneous sphere of two electron densities of diameters 106 and 203 Å, respectively, with the same center of symmetry. In addition, the casein submicelles exhibit overall an extremely low electron density which is consistent with the low packing density of the refined structures (Figs. 6 and 7). Such a low electron density may be interpreted as a high hydration value or a particle in which water can easily flow throughout the polypeptide chain or both. Many studies of several investigators (Farrell, 1988; Holt, 1992; Schmidt and Payens, 1976) also show that casein submicelles have a significantly higher hydration value than for globular proteins, i.e., 3–6 g water/g protein. Quantitative comparison of this submicelle structure with small-angle X-ray scattering results (Farrell *et al.*, 1990; Pessen *et al.*, 1991) has been achieved and is reported elsewhere (Kumosinski *et al.*, 1994b).

From our present structural study it would be reasonable to assume that the hydrophilic domains of α_{s1} -casein B within the synthetic submicelle

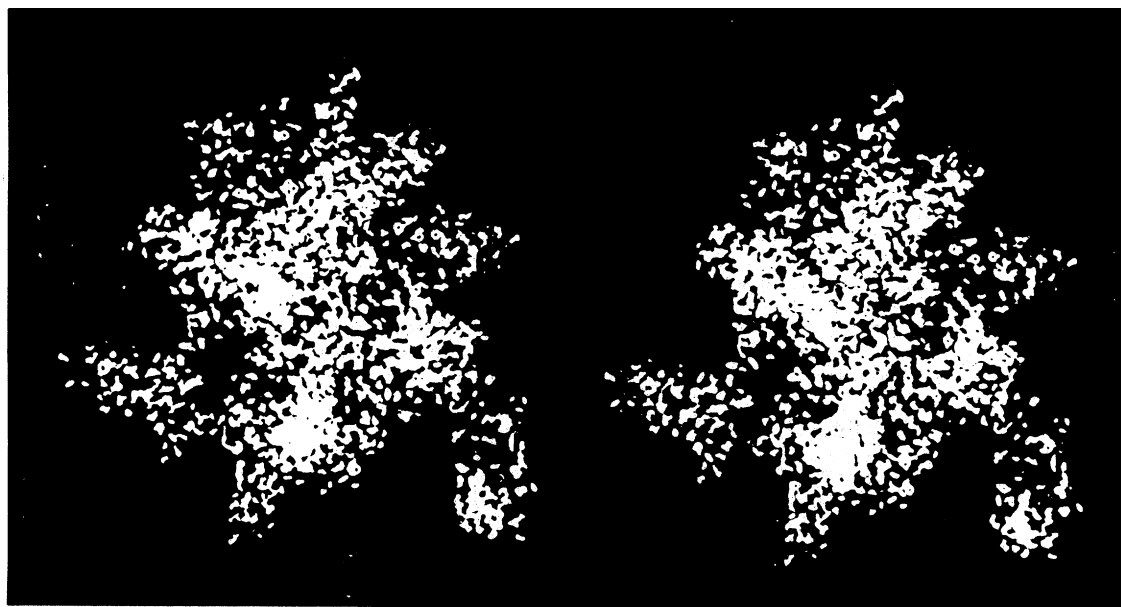


Fig. 7. Stereo view of refined casein symmetric submicelle structure with side chains and without backbone atoms. Same view as in Fig. 5B. Hydrophobic side chains are green, acidic ones red, and basic ones purple, while S-S or S-H groups are orange. β -Casein chymosin cleavage sites are orange and plasmin sites are red-orange.

structure (Fig. 3B) are potential interaction sites for colloid formation by self-association of the synthetic submicelle structure via calcium salt bridges. Such a colloidal matrix structure could easily allow for the temperature-induced hydrophobic association or disassociation of β -casein from that colloidal matrix structure. In fact, the remnant after cold dissociation would mimic the micelle framework postulated by Lin *et al.* (1972). It should be noted that the above is not a conclusive proof for the mechanism of submicelle and micelle formation. It is a working hypothesis, as is the structure, and is in need of a large amount of quantitative experiments to disprove, prove, or further refine this structure and mechanism. As with all such models, they are not an end in themselves but a springboard to further research.

REFERENCES

- Ali, A. E., Andrews, A. T., and Cheeseman, G. C. (1980). *J. Dairy Res.* **47**, 371-382.
- Baldwin, R. L., and Yang, Y. T. (1974). Enzymatic and metabolic changes in the development of lactation, in *Lactation: A Comprehensive Treatise* (Larson, B. L., and Smith, V. R., eds.), Academic Press, New York, pp. 349-355.
- Beeby, R. (1964). *Biochim. Biophys. Acta* **82**, 418-420.
- Berry, G. P., and Creamer, L. K. (1975). *Biochemistry* **14**, 3542-3545.
- Byler, D. M., and Farrell, H. M., Jr. (1989). *J. Dairy Sci.* **72**, 1719-1721.
- Byler, D. M., Farrell, H. M., Jr., and Susi, H. (1988). *J. Dairy Sci.* **71**, 2622-2628.
- Cohen, F. E., and Kuntz, I. D. (1989). In *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, J. D., ed.), Plenum Press, New York, p. 617.
- Cohen, F. E., Abarbanel, R. M., Kuntz, I. D., and Fletterick, R. J. (1986). *Biochemistry* **25**, 266-276.
- Creamer, L. K. (1976). *N. Z. J. Dairy Sci. Technol.* **11**, 30-39.
- Davies, D. T., and Law, A. J. R. (1980). *J. Dairy Res.* **47**, 83-90.
- Davies, D. T., and Law, A. J. R. (1983). *J. Dairy Res.* **50**, 67-75.
- Downey, W. K., and Murphy, R. F. (1970). *J. Dairy Res.* **37**, 361-372.
- Edsall, J. T., and McKenzie, H. A. (1983). *Adv. Biophys.* **16**, 53-183.
- Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell, H. M., Jr., Harwalkar, V. R., Jenness, R., and Whitney, R. McL. (1984). *J. Dairy Sci.* **67**, 1599-1631.
- Farrell, H. M. Jr. (1988). Physical equilibria: Proteins, in *Fundamentals of Dairy Chemistry*, 3rd ed. (Wong, N., ed.), Van Nostrand Reinhold, New York, pp. 461-510.
- Farrell, H. M., Jr., Kumosinski, T. F., Pulaski, P., and Thompson, M. P. (1988). *Arch. Biochem. Biophys.* **265**, 146-158.
- Farrell, H. M. Jr., Pessen, H., Brown, E. M., and Kumosinski, T. F. (1990). *J. Dairy Sci.* **73**, 3592-3601.
- Fox, P. F. (1981). *Neth. Milk Dairy J.* **35**, 233-255.
- Groves, M. L., Dower, H. J., and Farrell, H. M., Jr. (1992). *J. Protein Chem.* **11**, 21-28.
- Holt, C. (1992). *Adv. Protein Chem.* **43**, 63-113.
- Holt, C., and Sawyer, L. (1993). *J. Chem. Soc.* **89**, 2683-2690.
- Kumosinski, T. F., and Farrell, H. M., Jr. (1991). *J. Protein Chem.* **10**, 3-11.
- Kumosinski, T. F., Brown, E. M., and Farrell, H. M., Jr. (1993a). *J. Dairy Sci.* **76**, 931-945.
- Kumosinski, T. F., Brown, E. M., and Farrell, H. M., Jr. (1993b). *J. Dairy Sci.* **76**, 2507-2520.

- Kumosinski, T. F., Brown, E. M., and Farrell, H. M., Jr. (1994a). Three dimensional molecular modeling of bovine caseins: A refined energy minimized α_{s1} -casein structure, in *Molecular Modeling, From Virtual Tools to Real Problems* (Kumosinski, T. F., and Liebman, M. N., eds.), ACS Symposium Series, 576 Washington, DC, pp. 368-391.
- Kumosinski, T. F., King, G., and Farrell, H. M., Jr. (1994b). *J. Protein Chem.*, this issue.
- Kyře, J., and Doolittle, R. F. (1982). *J. Mol. Biol.* **157**, 105-132.
- Leaver, J., and Thomson, G. (1993) *Milchwissenschaft* **48**, 378-382.
- Lin, S. H. C., Leong, S. L., Dewan, R. K., Bloomfield, V. A., and Morr, C. V. (1972). *Biochemistry* **11**, 1818-1821.
- Mulvihill, D. M., and Fox, P. F. (1979). *J. Dairy Res.* **46**, 641-648.
- Noble, R. W., Jr., and Waugh, D. F. (1965). *J. Am. Chem. Soc.* **87**, 2236-2242.
- Noelken, M. E., Chang, P. J., and Kimmel, J. R. (1980). *Biochemistry* **19**, 1838-1843.
- Pepper, L. (1972). *Biochem. Biophys. Acta* **278**, 147-155.
- Pepper, L., and Farrell, H. M., Jr. (1982). *J. Dairy Sci.* **65**, 2259-2266.
- Pessen, H., Kumosinski, T. F., Farrell, H. M., Jr., and Brumberger, H. (1991). *Arch. Biochem. Biophys.* **284**, 133-142.
- Ribadeau Dumas, B., and Garnier, J. (1970). *J. Dairy Res.* **37**, 269-274.
- Rose, D. (1968). *J. Dairy Sci.* **51**, 1897-1902.
- Schmidt, D. G. (1982). Associations of caseins and casein micelle structure, in *Developments in Dairy Chemistry 1* (Fox, P. F., ed.), Applied Science, Essex, England, pp. 61-93.
- Schmidt, D. G., and Payens, T. A. J. (1976). Colloidal aspects of casein in surface and colloid science, Vol. 9 (Matijevic, E., ed.), Wiley, New York.
- Slattery, C. W., and Evard, R. (1973). *Biochem. Biophys. Acta* **317**, 529-538.
- Swaigood, H. E., Brunner, J. R., and Lillevik, H. A. (1964). *Biochemistry* **3**, 1616-1624.
- Van de Vroot, F. R., Ma, C.-Y., and Nakai, S. (1979). *Arch. Biochem. Biophys.* **195**, 596-603.
- Visser, S. (1981). *Neth. Milk Dairy J.* **35**, 65-78.
- Vreeman, H. J., Brinkhuis, J. A., and Van Der Spek, C. A. (1981). *Biophys. Chem.* **14**, 185-193.
- Walstra, P. (1990). *J. Dairy Sci.* **73**, 1965-1979.
- Waugh, D. F., and von Hippel, P. H. (1956). *J. Am. Chem. Soc.* **78**, 4576-4582.
- Waugh, D. F., Creamer, L. K., Slattery, C. W., and Dresdner, G. W. (1970). *Biochemistry* **9**, 786-795.
- Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Profeta, S., and Weiner, P. (1984). *J. Am. Chem. Soc.* **106**, 765-784.
- Weiner, S. J., Kollman, P. A., Nguyen, P. T., and Case, D. A. (1986). *J. Comput. Chem.* **7**, 230-252.
- Woychik, J. H., and Wondolowski, M. V. (1975). *J. Dairy Sci.* **52**, 1669-1670.
- Woychik, J. H., Kalan, E. B., and Noelken, M. E. (1966). *Biochemistry* **5**, 2276-2282.